

**A single residue in Ebola virus receptor NPC1 influences cellular host range in reptiles**

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Running title: Ebola virus host range determinants in reptiles

Abstract word count: 244

Text word count: [4280](#)

29

**Abstract**

30 Filoviruses are the causative agents of an increasing number of disease outbreaks in human  
31 populations, including the current unprecedented Ebola virus disease (EVD) outbreak in Western  
32 Africa. One obstacle to controlling these epidemics is our poor understanding of the host range  
33 of filoviruses and their natural reservoirs. Here, we investigated the role of the intracellular filo-  
34 virus receptor, Niemann-Pick C1 (NPC1) as a molecular determinant of Ebola virus (EBOV)  
35 host range at the cellular level. Whereas human cells can be infected by EBOV, a cell line de-  
36 rived from a Russell's viper (*Daboia russellii*) (VH-2) is resistant to infection in an NPC1-  
37 dependent manner. We found that VH-2 cells are resistant to EBOV infection because the Rus-  
38 sell's viper NPC1 ortholog bound poorly to the EBOV spike glycoprotein (GP). Analysis of pan-  
39 els of viper-human NPC1 chimeras and point mutants allowed us to identify a single amino acid  
40 residue in NPC1, at position 503, that bidirectionally influenced both its binding to EBOV GP as  
41 well as its viral receptor activity in cells. Significantly, this single residue change perturbed nei-  
42 ther NPC1's endosomal localization nor its housekeeping role in cellular cholesterol trafficking.  
43 Together with other recent work, these findings identify sequences in NPC1 that are important  
44 for viral receptor activity by virtue of their direct interaction with EBOV GP, and suggest that  
45 they may influence filovirus host range in nature. Broader surveys of *NPC1* orthologs from ver-  
46 tebrates may delineate additional sequence polymorphisms in this gene that control susceptibility  
47 to filovirus infection.

48

**Importance**

49 Identifying cellular factors that determine susceptibility to infection can help us understand how  
50 Ebola virus is transmitted. We asked if the EBOV receptor, Niemann-Pick C1 (NPC1) could ex-

plain why reptiles are resistant to EBOV infection. We demonstrate that cells derived from the Russell's viper are not susceptible to infection because EBOV cannot bind to viper NPC1. This resistance to infection can be mapped to a single amino acid residue in viper NPC1 that renders it unable to bind to EBOV GP. The newly solved structure of EBOV GP bound to NPC1 confirms our findings, revealing that this residue dips into the GP receptor-binding pocket, and is therefore critical to the binding interface. Consequently, this otherwise well conserved residue in vertebrate species influences the ability of reptilian NPC1s to bind to EBOV GP, thus affecting cellular host range.

### Introduction

Ebola virus (EBOV) is the causative agent of highly lethal zoonotic infections in humans and non-human primates in sub-Saharan Africa (1-3). Despite the emerging roles of EBOV and related members of the family *Filoviridae* (filoviruses) in human disease, our knowledge of the ecologic host range of these agents remains limited. Bats are thought to be important reservoirs for filoviruses; however, conclusive evidence in favor of this hypothesis has been obtained only for Marburg virus (MARV) and Ravn virus (RAVV), which were recently found to circulate in Egyptian rousettes (*Roussetus aegyptiacus*) (4-7).

Previous studies demonstrated that, whereas a broad range of mammalian and avian cell lines are susceptible to EBOV and/or MARV, all tested reptilian and amphibian lines are resistant to infection (8-10). These findings suggested the existence of one or more unknown determinants of filovirus host range. Although the determinants of filovirus infection and disease at the organismal level are likely to be complex, it is well established that interactions between viruses and cell-intrinsic host factors, such as entry receptors, can dictate host range. For example, ortholog-specific sequence variations in angiotensin-converting enzyme 2 (ACE2) and transfer-

74 rin receptor (TfR1) influence the host range of viruses for which they serve as receptors (severe  
75 acute respiratory syndrome-related coronaviruses (11, 12); New World mammarenaviruses, ca-  
76 nine parvoviruses, and murine mammary tumor virus (13-18), respectively). Jae and co-workers  
77 demonstrated that chicken cells are resistant to infection by an Old World arenavirus, Lassa vi-  
78 rus, because of a single amino acid difference in the chicken ortholog of its intracellular receptor,  
79 LAMP1 (19).

80 We and others recently demonstrated that Niemann-Pick C1 (NPC1), a large en-  
81 do/lysosomal membrane protein involved in cellular cholesterol trafficking, is an essential intra-  
82 cellular receptor for filovirus entry and infection (20-23). We also found that NPC1 could influ-  
83 ence the cellular host range of filoviruses—human NPC1 conferred susceptibility to filovirus en-  
84 try and infection when expressed in the non-permissive reptilian cell line VH-2, derived from a  
85 Russell’s viper (*Daboia russellii*) (22). In that study, however, we did not establish the molecular  
86 basis of the NPC1-dependent block to viral entry in VH-2 cells.

87 Recently, we found that a single amino acid residue (502) in the second luminal domain of  
88 NPC1, domain C, is under positive selection in bats, and controls susceptibility of bat cells to  
89 EBOV infection in a host species-dependent manner (24). Here, we demonstrate that an adjacent  
90 residue, 503, highly conserved in the domain C of NPC1, also influences EBOV host range in  
91 reptilian cells by controlling its activity as a filovirus receptor. The recently solved structure of  
92 EBOV GP bound to domain C shows that these two residues are in a loop that dips into the ex-  
93 posed receptor-binding site (25). Therefore, our findings identify a hotspot in NPC1 at the EBOV  
94 GP-binding interface that influences virus-receptor recognition and host cell susceptibility, sug-  
95 gesting evolutionary scenarios in which antagonism with filoviruses could sculpt host *NPC1*  
96 genes selectively, without compromising their ancient, and essential, function in cellular choles-



terol homeostasis.

## Results

**The second luminal domain of the Russell's viper NPC1 ortholog binds poorly to the Ebola virus glycoprotein.** We postulated that EBOV fails to enter and infect Russell's viper VH-2 cells because the EBOV entry glycoprotein, GP, cannot recognize the viper ortholog of the filovirus intracellular receptor, Niemann-Pick C1 (*DrNPC1*). We previously showed that the second luminal domain (C) of human NPC1 (*HsNPC1*) directly contacts a cleaved form of EBOV GP ( $GP_{CL}$ ), and that  $GP_{CL}$ -*HsNPC1* domain C binding is essential for filovirus entry (22, 23). Accordingly, we investigated the capacity of *DrNPC1* domain C to bind to  $GP_{CL}$  and support EBOV entry and infection.

We first used reverse-transcription PCR (RT-PCR) to isolate and sequence the *DrNPC1* domain C gene. Alignment of domain C amino acid sequences from *HsNPC1* and *DrNPC1* revealed a substantial degree of conservation (80% amino acid identity), with identical arrangements of cysteine residues and similar predicted secondary structures, suggesting a similar overall fold for both proteins (Fig. 1).

To facilitate *in vitro*  $GP_{CL}$ -NPC1 binding studies, we engineered a soluble form of *DrNPC1* domain C, as previously described for *HsNPC1* (22). Transfection of HEK 293T cells with this construct afforded the secretion of an extensively *N*-glycosylated form of *DrNPC1* domain C (Fig. 2A). As shown previously, purified *HsNPC1* domain C could bind to recombinant vesicular stomatitis Indiana virus particles bearing cleaved EBOV GP (rVSV- $GP_{CL}$ ) as measured by ELISA(22); however, no ELISA signal was apparent even at the highest concentration of *DrNPC1* domain C (Fig. 2B). Therefore, *DrNPC1* domain C, in contrast to its human

119 counterpart, recognizes the EBOV glycoprotein poorly or not at all.

120 ***Dr*NPC1 domain C can substitute for *Hs*NPC1 domain C in mediating endo/lysosomal cho-**  
121 **lesterol clearance but not EBOV entry and infection.** While the efficient secretion of the sol-  
122 uble, glycosylated *Dr*NPC1 domain C construct suggested that it was not misfolded, it was nev-  
123 ertheless conceivable that subtle structural aberrations rendered this protein biologically inactive.  
124 Accordingly, we assessed the capacity of *Dr*NPC1 domain C to support NPC1's best-established  
125 cellular function—clearance of unesterified cholesterol from endo/lysosomal compartments (Fig.  
126 3A-B). This activity requires the full-length NPC1 protein, including all three major luminal do-  
127 mains, A, C, and I. We therefore generated and tested an *Hs*NPC1 chimera in which domain C  
128 (residues 373–620) was seamlessly replaced with its viper counterpart (*Hs*NPC1–*Dr*C), and sta-  
129 bly expressed this construct in the NPC1-null Chinese hamster ovary (CHO) M12 cell line (26).  
130 As expected, immunostaining of NPC1 in a stable cell line expressing WT *Hs*NPC1 revealed a  
131 punctate, predominantly perinuclear distribution and 'donut-like' structure characteristic of  
132 NPC1's localization to the limiting membrane of late endosomes and lysosomes (Fig. 3A). This  
133 distribution could be readily contrasted with the filigree-like pattern obtained with  
134 *Hs*NPC1(I1061T), a point mutant that is susceptible to misfolding and is largely retained in the  
135 endoplasmic reticulum (27) (Fig. 3A). The behavior of the *Hs*NPC1–*Dr*C chimera in cells re-  
136 sembled that of WT *Hs*NPC1 and not *Hs*NPC1(I1061T), indicating that it too localizes to en-  
137 do/lysosomal compartments (Fig. 3A). This localization was further confirmed by transiently  
138 expressing the NPC1 constructs in a U2OS NPC1<sup>-/-</sup> cell line in which we could detect co-  
139 localization with the endo/lysosomal marker, LAMP1 (Fig. 3B) (Spence et al., in press). These  
140 results suggest that *Dr*NPC1 domain C is correctly folded and does not interfere with the correct  
141 folding and trafficking of full-length *Hs*NPC1–*Dr*C.

We next monitored the cholesterol clearance activity of each protein upon stable expression in the NPC1-null Chinese hamster ovary (CHO) M12 cell line (Fig. 3C). Filipin, a fluorescent probe for free cholesterol, extensively stained the cholesterol-laden endo/lysosomal compartments of the parental M12 cells, as shown previously (26). Ectopic *HsNPC1* expression could clear this accumulated cholesterol, as previously described (20), substantially reducing filipin staining. Remarkably, *HsNPC1-DrC* could rescue cholesterol clearance as efficiently as WT *HsNPC1* (Fig. 3C). These findings affirm that *DrNPC1* domain C is biologically active and competent to perform a major housekeeping function of its human counterpart, despite its divergence from the latter at 48 out of 248 amino acid positions.

Finally, we challenged M12 cell lines expressing WT *HsNPC1* or *HsNPC1-DrC* with authentic EBOV (Fig. 3D). Replacement of human domain C with its Russell's viper ortholog reduced EBOV infection by almost three orders of magnitude. Similar results were obtained in infections with rVSV-EBOV GP (Fig. 3E), confirming that the *DrNPC1* domain C-imposed infection block occurs at the viral entry step. Taken together, these observations afford two conclusions. First, the failure of *DrNPC1* to support EBOV entry and infection arises at least in part because its domain C cannot bind to EBOV GP<sub>CL</sub>. Second, one or more differences between the domain C sequences of *HsNPC1* and *DrNPC1* renders *DrNPC1* bereft of viral receptor activity without perturbing its normal function in cellular cholesterol homeostasis.

**Differences in *N*-glycosylation do not explain the defect in EBOV GP<sub>CL</sub>-*DrNPC1* domain C binding.** To uncover the molecular basis of *DrNPC1*'s defective viral receptor function, we engineered and tested a panel of mutant, soluble NPC1 domain C constructs in both *HsNPC1* and *DrNPC1* backgrounds. We first considered the possibility that one or more differences in *N*-linked glycosylation sites determines the *HsNPC1-DrNPC1* difference, because it is either re-

quired for GP<sub>CL</sub>-*HsNPC1* binding or deleterious for GP<sub>CL</sub>-*DrNPC1* binding (Fig. 4). Six sequons are conserved between the two proteins, but *DrNPC1* and *HsNPC1* domain C contain two and one unique sequons, respectively. Accordingly, we generated soluble domain C proteins containing or lacking each unique sequon and tested these putative gain-of-function and loss-of-function mutants for binding to EBOV GP<sub>CL</sub>. 'Humanized' *DrNPC1* domain C proteins engineered to lack their unique sequons at positions 414 or 498 (*HsNPC1* numbering) or to gain the sequon at position 598 of *HsNPC1* remained defective at EBOV GP<sub>CL</sub> binding in the ELISA. Conversely, *HsNPC1* domain C proteins engineered to resemble *DrNPC1* at each of these three positions remained fully competent to bind to EBOV GP<sub>CL</sub>. Therefore, differences in *N*-linked glycosylation between the domain Cs of *HsNPC1* and *DrNPC1* do not account for the defective EBOV receptor activity of *DrNPC1*.

**A single point mutation renders *DrNPC1* domain C competent to bind to EBOV GP<sub>CL</sub>.** Having ruled out a role for variations in *N*-glycosylation, we next adopted a systematic approach to identify determinative sequences in NPC1 domain C. We expressed a series of soluble *HsNPC1*-*DrNPC1* domain C chimeras, and measured their activity in the GP<sub>CL</sub>-binding ELISA (Fig. 5). However only chimera 2, *DrNPC1* domain C containing *HsNPC1* residues 476–536, afforded GP<sub>CL</sub>-NPC1 binding (Fig. 5A).

Chimera 2 introduced 14 Russell's viper→human amino acid changes into *DrNPC1*. To further dissect their roles, we generated and tested three additional chimeras containing subsets of these amino acid changes (chimeras 4–6, Fig. 5B) in the GP<sub>CL</sub> binding ELISA. The sub-region chimera 5 fully reconstituted GP<sub>CL</sub>-*DrNPC1* domain C binding, providing evidence that one or more of the 6 *HsNPC1* residues in this construct confers gain of function to *DrNPC1*.

To assess the individual contributions of the six Russell's viper→human amino acid changes in chimera 5, we separately introduced these changes into soluble *Dr*NPC1 domain C, and tested the capacity of each point mutant to bind to EBOV GP<sub>CL</sub> by ELISA (Fig. 6). A single conservative mutation, Tyr 503→Phe, fully restored GP<sub>CL</sub>-*Dr*NPC1 domain C binding, whereas the other 5 mutations had no discernible effect. Thus, the presence of Tyr instead of Phe at position 503 appears to completely explain the failure of *Dr*NPC1 to bind to EBOV GP<sub>CL</sub>.

**Phe ! Tyr sequence change at residue 503 controls NPC1's function as an EBOV entry receptor without affecting its housekeeping function.** We postulated that the Phe↔Tyr sequence change at residue 503 might influence EBOV GP<sub>CL</sub>-NPC1 binding in a bidirectional manner. Accordingly, we expressed and purified the reciprocal *Dr*NPC1(Y503F) and *Hs*NPC1(F503Y) domain C mutants, and tested them in the GP<sub>CL</sub> binding ELISA (Fig. 7). Purified *Dr*NPC1(Y503F) domain C bound almost as well as its human counterpart to EBOV GP<sub>CL</sub> (binding EC<sub>50</sub>≈3 nM [Russell's viper] vs. 0.5 nM [human]). Conversely, no detectable GP<sub>CL</sub> binding was obtained with the *Hs*NPC1(F503Y) domain C protein (EC<sub>50</sub>> 1 μM).

To examine the consequences of the 503(Phe↔Tyr) sequence change for the cellular and viral receptor functions of NPC1, we introduced the F503Y and Y503F mutations into full-length *Hs*NPC1 and the seamless *Hs*NPC1-*Dr*C chimera, respectively, and expressed them stably in M12 NPC1-null cells (Fig. 8). Immunostaining of NPC1 in these cell lines revealed punctate, perinuclear staining resembling that observed for WT *Hs*NPC1 and *Hs*NPC1-*Dr*C (Figs. 8A and 3A), as well as colocalization with LAMP1 in transiently transfected U2OS NPC1<sup>-/-</sup> cell lines (Figs. 8B and 3B). Furthermore, filipin staining showed little or no cholesterol accumulation in cells expressing *Hs*NPC1(F503Y) or *Hs*NPC1-*Dr*C(Y503F) (Fig. 8B). Therefore, the F503Y and

209 Y503F mutations do not substantially affect the folding, endosomal delivery, and cholesterol  
210 clearance function of NPC1.

211 Finally, we challenged cell lines expressing the 503(Phe↔Tyr) NPC1 mutants with  
212 authentic EBOV and rVSV-EBOV-GP (Fig. 8C). The capacities of both authentic and surrogate  
213 viruses to enter and infect these cells were fully congruent with the results of the GP binding ex-  
214 periments. The viper→human Y503F mutation afforded the complete restoration of viral infec-  
215 tion in cells expressing the *HsNPC1-DrC* chimera ( $\approx 100,000\%$  increase). Reciprocally, the hu-  
216 man→viper F503Y mutation reduced viral infection in cells expressing *HsNPC1* by  $\approx 99\%$ . Thus,  
217 the infection data correlate with the GP<sub>CL</sub>-domain C binding data, demonstrating that switching  
218 the residue at position 503 changes the ability of human and Russell's viper NPC1 domain C to  
219 bind EBOV GP<sub>CL</sub>, thereby determining the ability of these NPC1 proteins to be used as EBOV  
220 receptors.

221 **A bulky, hydrophobic amino acid residue at position 503 favors EBOV GP<sub>CL</sub>-NPC1 do-**  
222 **main C binding.** To determine the mechanism by which the change in NPC1 residue 503 con-  
223 trols binding of *HsNPC1* to EBOV GP<sub>CL</sub>, we engineered a series of NPC1 domain C proteins  
224 bearing amino acid residues with divergent physicochemical properties at position 503. Exami-  
225 nation of these mutants by GP<sub>CL</sub> binding ELISA revealed that binding avidity generally correlat-  
226 ed with amino acid size and polarity (Fig. 9A). Specifically, residues with bulky, hydrophobic  
227 side chains (Leu, Trp) afforded GP<sub>CL</sub>-NPC1 binding at WT levels, whereas residues with polar  
228 side chains (Asp, His, Ser) completely abrogated binding. Binding was greatly reduced, but de-  
229 tectable, with Ala and Thr at residue 503. The recently solved structure of EBOV GP<sub>CL</sub> bound to  
230 NPC1 domain C shows that this residue inserts into the hydrophobic trough of EBOV GP<sub>CL</sub> (28,  
231 29) similar to residue F225 of the EBOV glycan cap (Fig. 9B, 9C, (25)).

**The tyrosine residue at position 503 controls EBOV GP<sub>CL</sub> binding function in reptile NPC1 domain C orthologs.** Finally, we asked if our findings had implications for host cell range in other vertebrates, especially reptiles, which appear to be refractory to infection by EBOV (8, 30). An alignment of available NPC1 domain C sequences from a panel of vertebrate species revealed that, although there exists a number of differences in amino acid sequence around residue 503, the Phe at this position is itself very well conserved among vertebrates, with only two *NPC1* orthologs—those of the Russell’s viper and king cobra (*Ophiophagus hannah*)— encoding a Tyr at this position (Fig. 10A). Interestingly, the predicted NPC1 polypeptide sequences of two additional snakes, the Burmese python (*Python bivittatus*) and the common garter snake (*Thamnophis sirtalis*), encode a Phe at position 503 (Fig. 10A). To investigate the GP<sub>CL</sub>-binding capacities of the snake NPC1 orthologs, we expressed and purified soluble NPC1 domain C proteins for the king cobra and Burmese python, and tested them for binding to EBOV GP<sub>CL</sub>. The capacity of these proteins to bind to EBOV GP<sub>CL</sub> was concordant with the identity of the residue at NPC1 codon 503. Thus, king cobra NPC1 domain C (Tyr 503) resembled viper NPC1 domain C in its inability to bind to EBOV GP<sub>CL</sub>, whereas Burmese python NPC1 domain C (503 Phe) readily bound to EBOV GP<sub>CL</sub> (Fig. 10B). We tested two more reptilian NPC1 orthologs—from Chinese softshell turtle and Carolina anole (both encoding Phe at position 503), and found that they could all bind to EBOV GP<sub>CL</sub> (Fig. 10C). These findings provide additional evidence that *NPC1* residue 503 influences the cellular host range of EBOV at the level of virus-receptor recognition, and raise the possibility that sequence differences at this position influence the susceptibility of reptiles to filovirus infection in nature.

## Discussion

The essential entry receptor NPC1 is the first known molecular determinant of the cellular host range of EBOV and other filoviruses (24). In this study, we uncover one mechanism by which NPC1 imposes a species-specific barrier to EBOV infection. We show that reptilian cells derived from the Russell's viper, *Daboia russellii*, are largely resistant to EBOV entry and infection because of the presence of a Tyr residue at position 503 in NPC1, whereas the NPC1 orthologs of most other types of animals, include humans, carry a highly conserved Phe residue. Unexpectedly, toggling this residue between Phe and Tyr in either human or viper NPC1 backgrounds switched each protein's ability to act as an EBOV receptor. NPC1's crucial housekeeping function—distribution of cholesterol from the endo/lysosomal compartment to other cellular membranes—remained unaffected by these changes. Thus, our work identifies a genetic determinant in NPC1 that controls its viral receptor function, and consequently, host susceptibility to EBOV infection, in a manner that is selective, yet transferable between highly divergent NPC1 orthologs.

The determinative Phe 503→Tyr change is located in NPC1's second luminal domain (C), which directly binds to a cleaved form of the EBOV entry glycoprotein (GP<sub>CL</sub>) during viral entry (22, 23, 25). Here, we found that Phe 503→Tyr renders cells non-permissive to EBOV infection because it reduces the apparent binding affinity of GP<sub>CL</sub> for NPC1 domain C by more than 1000-fold. What mechanism might account for this extraordinary effect of a single hydroxyl group on virus-receptor interaction?

The recently solved structure of the EBOV GP<sub>CL</sub> bound to NPC1 domain C reveals that Phe 503 in human NPC1 domain C inserts deeply into the hydrophobic GP<sub>CL</sub> trough during GP-NPC1 interaction, in a manner that resembles the interaction of Phe 225 in the GP glycan cap with the GP<sub>CL</sub> trough in uncleaved GP (Fig. 9B) (25). The introduction, at position 503, of a po-



lar hydroxyl group (Tyr) or other polar side chains (Asp, His, Ser, Gly) is likely to be energetically unfavorable, thereby reducing the affinity of GP<sub>CL</sub>-NPC1 binding.

We recently demonstrated that the residue 502 in NPC1 was under positive selection in bats, and was responsible for the reduced susceptibility of African straw-colored fruit bat cells to EBOV infection (24). Since none of the bat species encodes a Tyr at position 503 in NPC1, there was no observed signature of positive selection at this residue. The structure rationalizes the effect of these residues on GP-NPC1 binding, as both are located in the  $\alpha 4$ – $\alpha 5$  loop of NPC1 domain C that directly interacts with EBOV GP<sub>CL</sub> ('loop 2'; (25)).

It is unclear what relationships, if any, exist (or have existed) in nature between filoviruses and snakes or other reptiles. Experimental infections of wild-caught reptiles and amphibians by Swanepoel and colleagues (30) showed a general refractoriness to EBOV infection or replication, but minimal titers were recovered on a few occasions from the brown house snake (*Lamprophis fuliginosus*). Following outbreaks of the Ebola relative, Marburg virus (MARV) at the mine in Kitaka Cave, the nearby “Python cave” in Queen Elizabeth National Park in Uganda (31, 32) and the Goroubwa Mine in the Democratic Republic of Congo (33), a number of Egyptian fruit bats found to be infected with MARV (5, 6, 34). Unfortunately, though the African Rock python (*Python sebae*) and forest cobras (*Naja melanoleuca*) are part of the fauna in these locations, there were no reports on investigations on snakes from these caves for filovirus infection(33), (6). Nevertheless, our finding that two snake NPC1 orthologs are nonpermissive to filovirus entry and infection due to a single amino acid change leads us to speculate that this change was an adaptation to reduce infection by a filovirus, thereby increasing host survivability. More extensive wildlife sampling coupled with genetic and functional analysis of host-virus interactions associated with filovirus infection may uncover additional evidence for evolutionary arms

300 races between filoviruses and multiple types of animals (bats, reptiles, rodents).

## 301 **Materials and Methods:**

### 302 **Cells**

303 Vero grivet and HEK 293T cells were maintained in high-glucose Dulbecco's modified Eagle  
304 medium (DMEM; Thermo Fisher Scientific, Waltham MA) supplemented with 10% fetal bovine  
305 serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Thermo  
306 Fisher Scientific) at 37°C and 5% CO<sub>2</sub>. Chinese hamster ovary (CHO) cells were maintained in  
307 DMEM-Ham's F-12 medium (50/50 mix) (Thermo Fisher Scientific) supplemented with 10%  
308 FBS at 37°C and 5% CO<sub>2</sub>. Cell lines were generated by retroviral transduction system, as previ-  
309 ously described (22), to stably overexpress the NPC1 constructs in CHO-M12 cells, which con-  
310 tain a deletion in the NPC1 locus (26) (transient transfections of NPC1 constructs + LAMP1 for  
311 colocalization experiments). FreeStyle™ 293-F cells were maintained in GIBCO FreeStyle™  
312 293 expression medium (Thermo Fisher Scientific) at 37°C and 8% CO<sub>2</sub>.

### 314 **NPC1 constructs**

315 NPC1 domain C sequences (residues 373 to 620) flanked by sequences that form anti-parallel  
316 coiled coils as previously described (35), were cloned into the pcDNA3.1(+) vector. Constructs  
317 made were included glycosylation mutants in *HsNPC1* domain C: L414N+D416T,  
318 K498N+G500S and N598A, while those in *DrNPC1* domain C: N414A, N498A and R600T.  
319 *DrNPC1* domain C chimeras were made by substituting these residues for human residues: 373-  
320 475 (chimera 1), 476-536 (chimera 2), 537-620 (chimera 3), 493-502 (chimera 4), 502-512 (chi-  
321 mera 5) and 513-522 (chimera 6), and point mutations made were E502D, Y503F, I505V,  
322 H506Y, F509Y and S511T. The constructs were then transiently transfected into HEK 293T

cells and the supernatant with secreted protein was harvested after 72h and used in ELISA assays. Purified proteins were made by transfecting FreeStyle™ 293-F cells in suspension, harvested 72 h post-transfection and purifying by incubation with His-60 Nickel resin. The proteins were eluted at 500mM imidazole and pH 7.6, and dialyzed into 50mM MES, 150mM NaCl, pH5.5. Domain C chimeras in the full length NPC1 were generated by seamlessly replacing the domain C sequences in the *HsNPC1*. The constructs were subcloned into the pBABE-puro retroviral vector and stably transfected into CHO-M12 cells by retroviral transduction, as previously described (22). All constructs possessed *N*-terminal flag tags.

### **VSV pseudotype infections**

Replication-incompetent vesicular stomatitis Indiana virus (VSV) pseudotypes encoding eGFP in the first position and EBOV GP in place of VSV G were made as previously described (9, 36). EBOV GPΔMuc matches the EBOV/H.sapiens-tc/COD/1976/Yambuku-Mayinga isolate amino acid sequence (GenBank accession number AF086833), but lacks the mucin-like domain (Δ309–489; ΔMuc) (37). Unless otherwise indicated, viruses were titrated on Vero grivet monkey cells by manual counting of eGFP-positive cells. Cleaved EBOV GP (GP<sub>CL</sub>) was generated *in vitro* using the bacterial protease thermolysin (250μg/mL) (Sigma-Aldrich, St Louis, MO) for 1h at 37°C as described previously (38, 39), and the reaction was stopped by adding the metalloprotease inhibitor phosphoramidon (1mM) (Sigma-Aldrich).

### **Authentic Ebola virus infections**

CHO cells, seeded in black Cellcoat® 96 well plates (Greiner Bio-One North America, Monroe, NC) were incubated with Ebola virus/H.sapiens-tc/COD/1995/Kikwit-9510621 at the indicated

multiplicity of infection in a biosafety level 4 (BSL-4) laboratory located at USAMRIID. Following 1h absorption, virus inoculum was removed and cells were washed once with PBS. Cells were then incubated at 37°C, 5% CO<sub>2</sub>, 80% humidity for 72h, at which time, the cells were washed once with PBS and submerged in 10% formalin prior to removal from the BSL-4 laboratory. Formalin was removed and cells were washed 3 times with PBS. Cells were blocked by adding 3% BSA/PBS to each well and incubating at 37°C for 2h. Cells were incubated with EBOV GP-specific mAb KZ52, diluted to 1µg/mL in 3% BSA/PBS, at room temperature for 2h. Cells were washed 3 times with PBS prior to addition of goat anti-human IgG-AlexaFluor 488 (Thermo Fisher Scientific) secondary antibody. Following 1h incubation with secondary antibody, cells were washed 3 times prior to addition of Hoechst 33342 (Thermo Fisher Scientific) diluted in PBS. Cells were imaged and percent of virus infected cells calculated using the Operetta High Content Imaging System (PerkinElmer, Waltham, MA) and Harmony® High Content Imaging and Analysis Software (PerkinElmer).

#### **GP<sub>CL</sub> –NPC1 domain C capture ELISA**

Normalization of NPC1 domain C supernatants and proteins was carried out as previously described (24): resolving on SDS-PAGE followed by immunoblotting with anti-flag primary antibody (Sigma Aldrich) and anti-mouse Alexa-680 secondary antibody (Thermo Fisher Scientific), and quantified on the LI-COR Odyssey Imager (LI-COR Biosciences, Lincoln, NE). Capture ELISAs were also performed as previously described (22, 24). Briefly, high-binding 96-well ELISA plates (Corning, Corning, NY) were coated with KZ52 (40) (2µg/mL in PBS) and then blocked using PBS containing 3% bovine serum albumin (PBSA). Pseudotyped EBOV was cleaved with thermolysin (250µg/mL) at 37°C for 1h and captured on the plate. Unbound virus

was washed off and serial dilutions of either flag-tagged purified soluble NPC1 domain C (domain C; 0–40 µg/mL), or supernatants from transient transfections of the NPC1 constructs on HEK 293T cells were added. Bound domain C was detected by a horseradish conjugated anti-flag antibody, and Ultra-TMB substrate (ThermoFisher). EC<sub>50</sub> values were calculated from binding curves generated by non-linear regression analysis using Prism (GraphPad Software, La Jolla CA). Binding ELISAs were done in duplicate, and in at least two independent experiments. All incubation steps were done at 37°C for 1 hour or at 4°C overnight.

### **Immunofluorescence**

Imaging was performed in U2OS or CHO cells grown on 12-mm coverslips and fixed with 4% paraformaldehyde. For antibody staining, the coverslips were incubated with an anti-flag antibody (Sigma Aldrich) in PBS containing 0.1% Triton X-100 and 1% BSA. Detection was by incubation with Alexa 488-conjugated secondary antibodies (Thermo Fisher Scientific). For filipin staining, the coverslips were stained with 50 µg/mL of *Streptomyces filipensis* filipin III complex (Sigma Aldrich) in PBS for 1h. Coverslips were mounted on glass slides using ProLong antifade reagent (Thermo Fisher Scientific) and images were acquired with an inverted fluorescence microscope equipped with a 63X high-numerical aperture oil objective.

### **Acknowledgments**

We thank Tyler Krause, Cecelia Harold, and Tanwee Alkutkar for excellent technical support. We are grateful to Jens H. Kuhn for comments on a preliminary version of this manuscript. We are grateful to T. Y. Chang for his gift of CHO M12 cells.

Supported by grants from the U.S. National Institutes of Health (AI101436 to K.C.), and

the U.S. Defense Threat Reduction Agency (CB3948 to J.M.D.). K.C. is additionally supported by a Harold and Muriel Block Faculty Scholarship and an Irma T. Hirschl/Monique Weill-Caulier Research Award at the Albert Einstein College of Medicine. Opinions, conclusions, interpretations, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Department of the Army and the U.S. Department of Defense.

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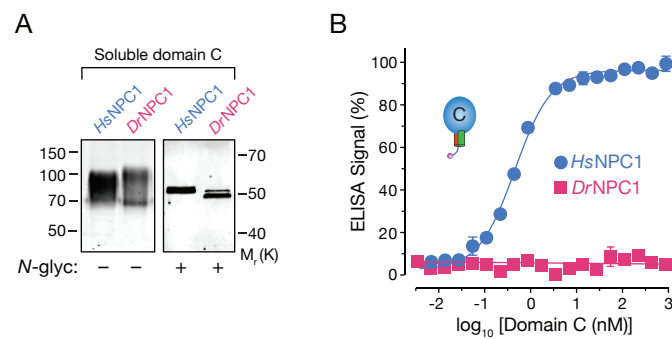
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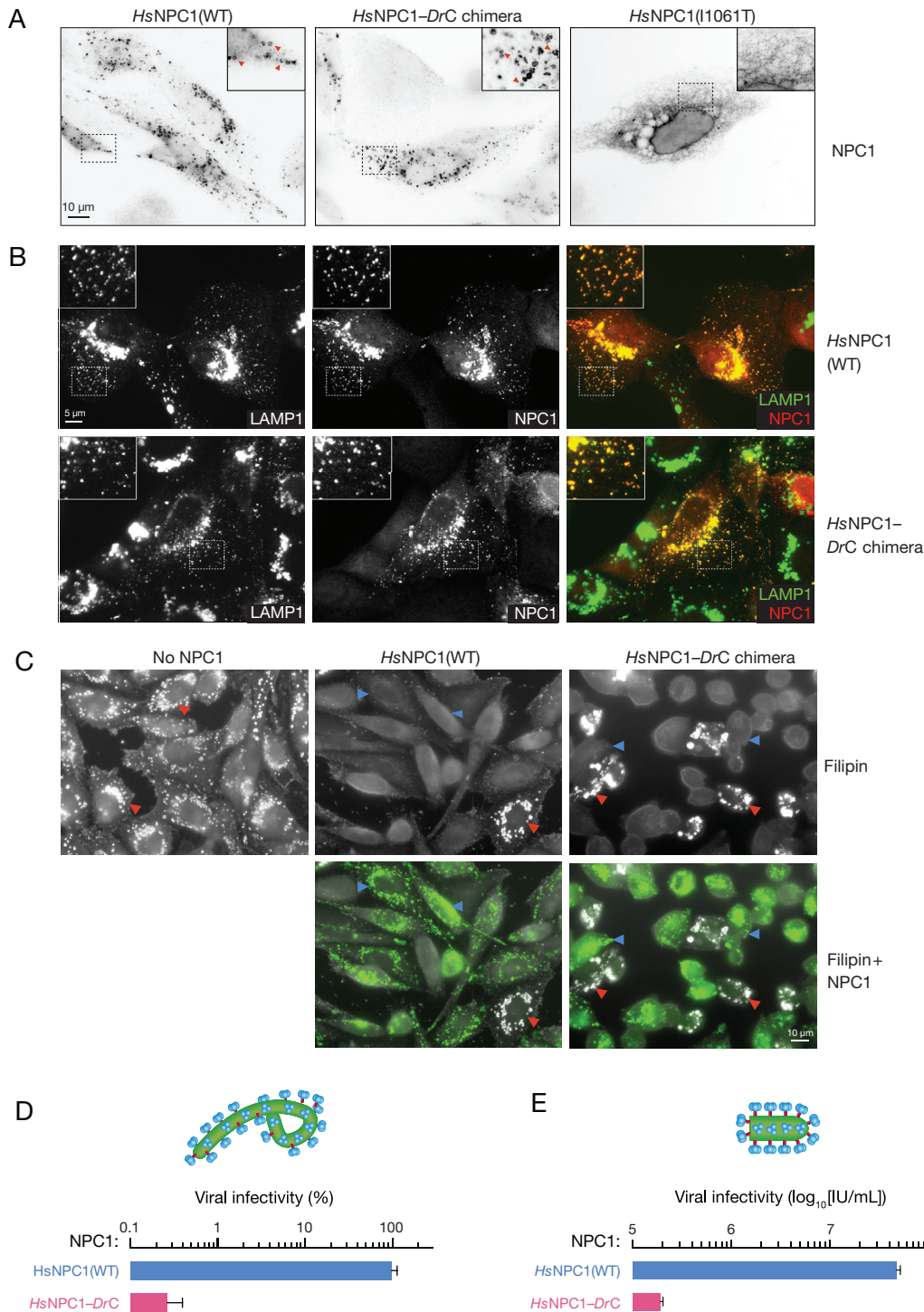


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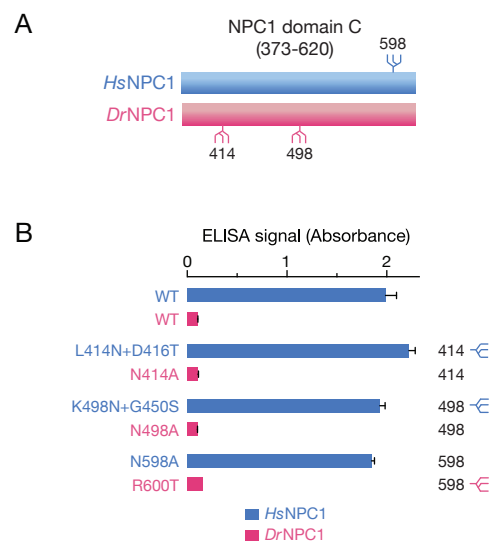




**Fig 2: Both *HsNPC1* and *DrNPC1* domain C proteins are expressed and secreted but bind differentially to EBOV GP<sub>CL</sub>.** (A) Soluble forms of the NPC1 domain C proteins from human (*HsNPC1*) and Russell's viper (*DrNPC1*) were expressed in FreeStyle™ 293-F cells and purified by nickel-affinity chromatography. Equal concentrations were resolved by anti-flag immunostaining. Left, no treatment. Right, treated with protein *N*-glycosidase F. (B) The two NPC1 domain C proteins were tested in an ELISA for binding to EBOV GP<sub>CL</sub>. VSV-EBOV GP viruses were cleaved with thermolysin (250µg/mL) and captured on an ELISA plate using the monoclonal antibody KZ52. Serial dilutions of either *HsNPC1* or *DrNPC1* domain C proteins were added, and binding to GP<sub>CL</sub> was detected by anti-flag antibody.



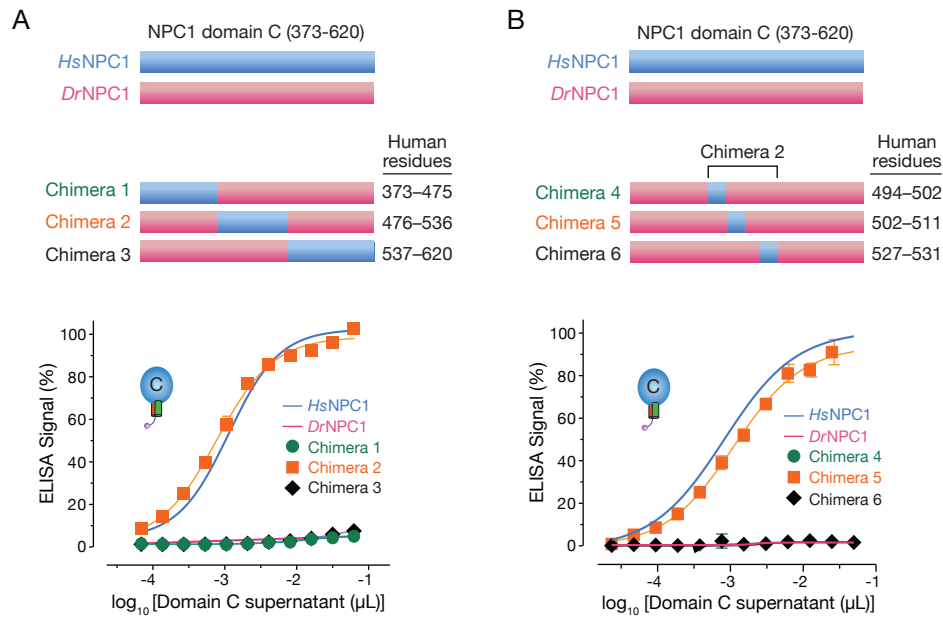
**Fig 3: *HsNPC1-DrC* chimera is functional at cholesterol clearance from lysosomes, but does not support EBOV entry and infection.** (A) Distribution of full length NPC1 constructs stably expressed in a CHO cell line that lacks a functional NPC1 (CHO-M12). *HsNPC1* and the human-viper chimera (*HsNPC1-DrC*) display a typical late endosomal/lysosomal localization pattern. In contrast, the mutant *HsNPC1(I1061T)* is retained in the endoplasmic reticulum. (B) NPC1 constructs from (A) immunostained with anti-flag antibody (red), colocalize with the lysosomal marker, LAMP1 (green) when transiently expressed in U2OS cells. (C) CHO-M12 cells stably expressing either *HsNPC1* WT or *HsNPC1-DrC* were stained with filipin to visualize unesterified cholesterol. Top panel, filipin staining. Cholesterol-laden cells are marked with red arrows. Blue arrows indicate cells that are functional at cholesterol clearance. Bottom panel, cells immunostained with anti-flag antibody for NPC1 expression (green). (D) Infection of cells from (C) by authentic EBOV (MOI of 10), scored 72h post infection and normalized to infection on *HsNPC1(WT)*. (E) Infection of cells from (C) by VSV-EBOV GP calculated by manual counting of eGFP positive cells. IU/mL, infectious units per mL.



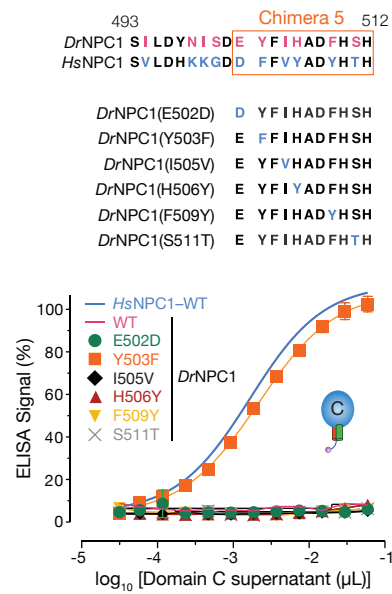
**Fig 4: *N*-glycosylation of NPC1 domain C does not affect EBOV GP<sub>CL</sub> binding.**

(A) Location of the three unique sequons in *HsNPC1* vs. *DrNPC1* domain C.

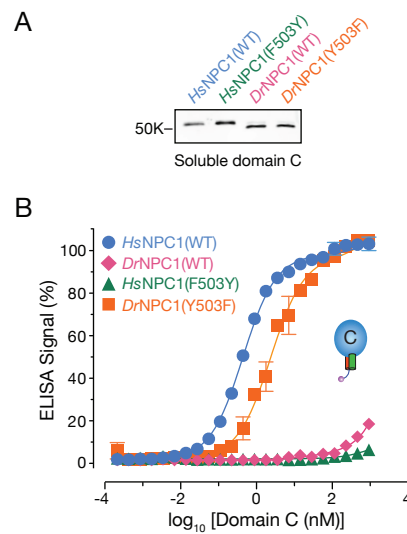
(B) Glycosylation mutants were made in both *HsNPC1* (losing sequon at position 598 and gaining sequons at position 414 and 498) and *DrNPC1* (losing sequons at position 414 and 498 and gaining sequon at position 598). Domain C proteins were expressed in HEK 293T cells and tested for EBOV GP<sub>CL</sub> binding by ELISA



**Fig 5: Middle region of *HsNPC1* domain C confers binding ability to *DrNPC1*.** (A) Chimeras were engineered by replacing *DrNPC1* domain C sequences with human sequences 373–475 (chimera 1), 476–536 (chimera 2) or 537–620 (chimera 3). The chimeras were expressed in HEK 293T cells and tested for EBOV GP<sub>CL</sub> binding by ELISA. (B) Further dissection of chimera 2 was done by replacing smaller subsets of *DrNPC1* with human residues: 494–502 (chimera 4), 502–511 (chimera 5) and 527–531 (chimera 6). Chimeric NPC1 domain C proteins were tested as in (A).

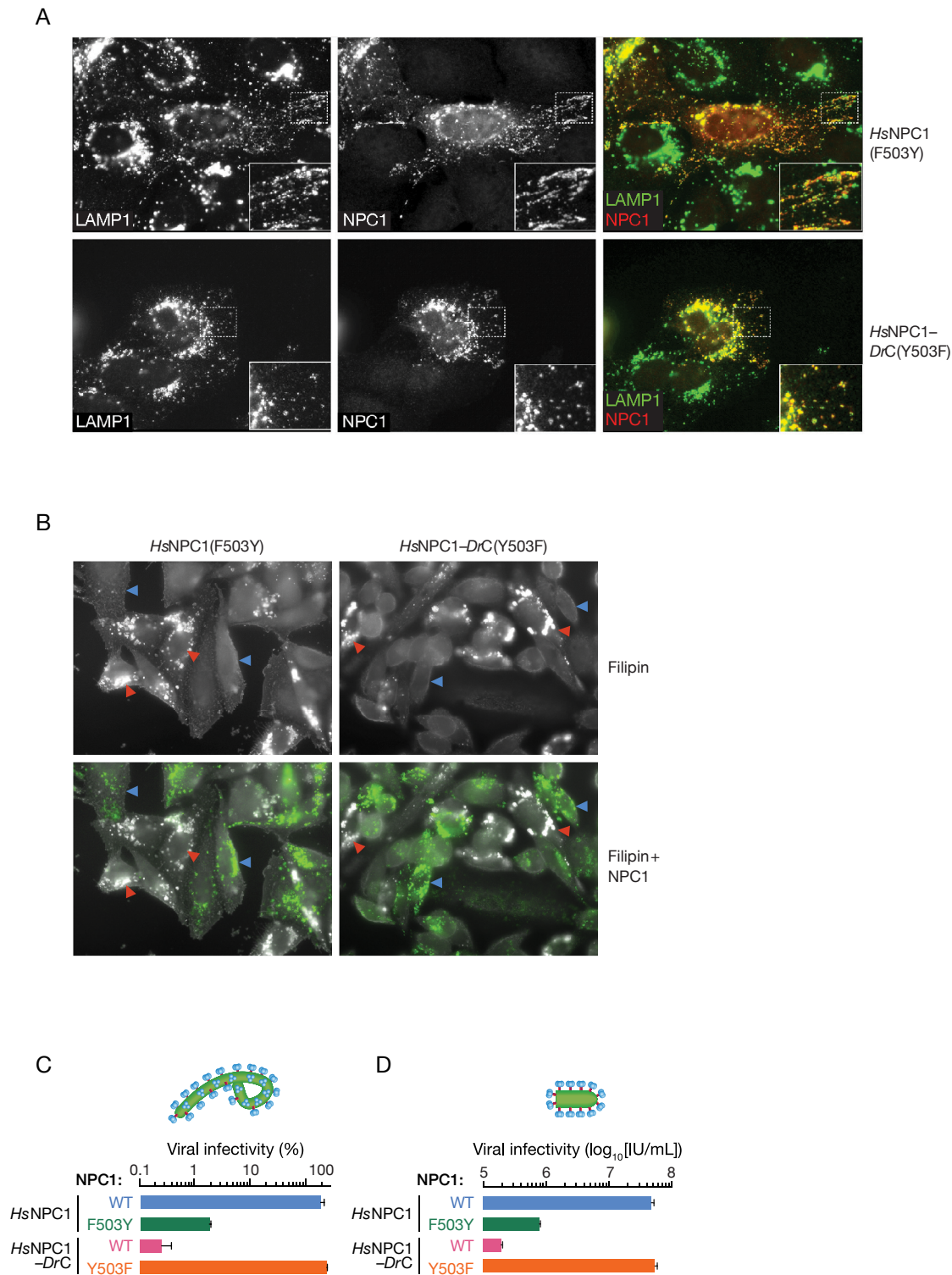


**Fig 6: A single amino acid change renders *DrNPC1* domain C fully competent to bind EBOV GP<sub>CL</sub>.** Chimera 5 contains 6 amino acid differences between *DrNPC1* and *HsNPC1* domain C. Point mutations were made in the *DrNPC1* domain C by switching the viper amino acid residue at each of these positions to the corresponding human residue: E502D, Y503F, I505V, H506Y, F509Y and S511T. The point mutants were expressed in HEK 293T cells and tested for EBOV GP<sub>CL</sub> binding by ELISA.

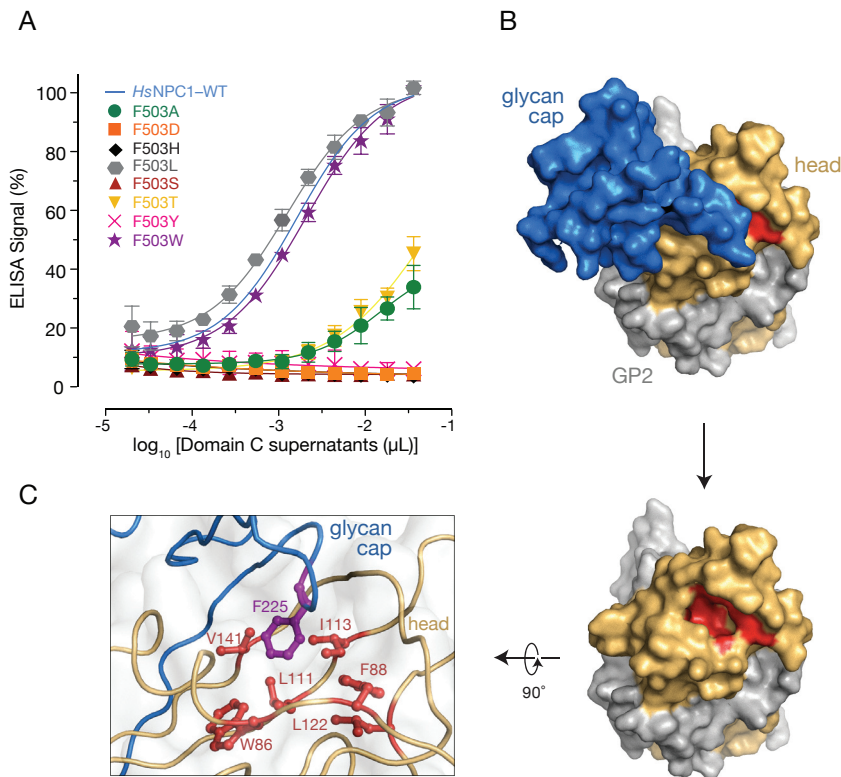


**Fig 7: NPC1 residue 503 bidirectionally alters domain C's capacity to bind EBOV GP<sub>CL</sub>.** (A) *HsNPC1* and *DrNPC1* domain C proteins bearing point mutations at residue 503 (*HsNPC1*, F503Y; *DrNPC1*, Y503F) were expressed and purified. (B) Serial dilutions of equivalent amounts of purified NPC1 domain C proteins were tested for EBOV GPCL binding by ELISA.

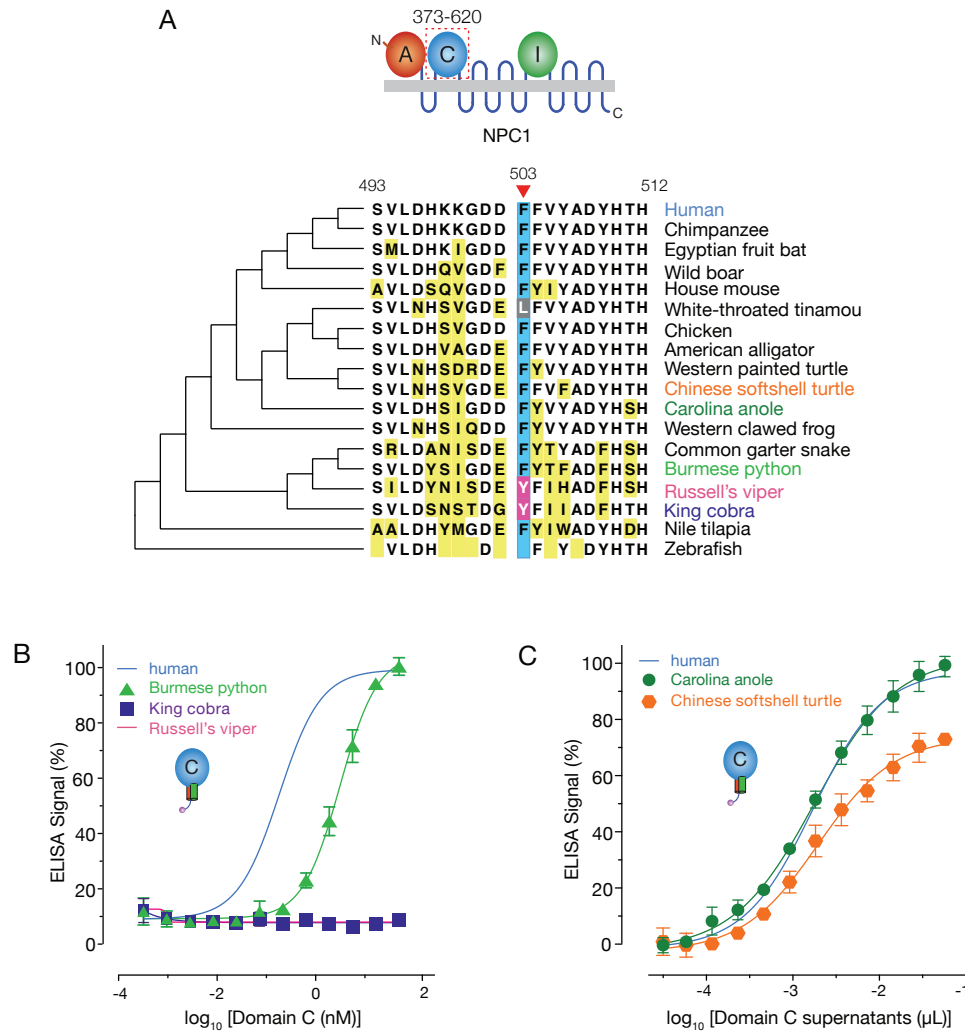




**Fig 8: Residue 503 influences the capacity of full-length NPC1 to support EBOV entry and infection** (A) Point mutations at residue 503 were introduced into *HsNPC1* and the chimera *HsNPC1-DrC* (F503Y and Y503F, respectively), and these constructs were transiently expressed in U2OS cells. NPC1 (green) and a lysosomal marker, LAMP1 (red), were visualized by immunofluorescence microscopy. (B) NPC1-deficient CHO-M12 cells stably expressing either *HsNPC1*(F503Y) or *HsNPC1-DrC* (Y503F) were stained with filipin to visualize unesterified cholesterol. Top panel, filipin staining. Cholesterol-laden cells are marked with red arrows. Blue arrows indicate cells that are functional at cholesterol clearance. Bottom panel, cells immunostained with anti-flag antibody for NPC1 expression (green). (C) CHO-M12 cells stably expressing the NPC1 proteins indicated were exposed to authentic virus (MOI of 3), scored at 72h post infection and normalized to *HsNPC1* WT infectivity. (D) Infection by VSV-EBOV GP, calculated by manual counting of eGFP positive cells. IU/mL, infectious units per mL



**Fig 9: A bulky, hydrophobic residue is required at position 503.** (A) Phe at NPC1 residue 503 was mutated to Ala, Asp, His, Leu, Ser, Thr or Trp and tested for binding to EBOV GP<sub>CL</sub> by ELISA. (B) Structure of EBOV GP monomer with GP1 in orange and GP2 in grey, and glycan cap (blue) occluding the NPC1-binding site (red) (PDB ID: 3CSY [27]). Proteolytic removal of the glycan cap and mucin domain (not shown) in host cell endosomes unmask this site. (C) Interaction between Phe 225 of the glycan cap and residues Trp86, Phe88, Leu111, Ile113, Leu122 and Val141 in the GP1 hydrophobic trough.



**Fig 10: The Tyr residue at NPC1 position 503 is unique to the Russell's viper and king cobra NPC1 orthologs.** (A) Alignment of sequences flanking residue 503 (red arrow) in domain C from divergent NPC1 orthologs. Phe 503 is shaded blue. Residues different from the human sequence are highlighted in yellow. (B) Binding of NPC1 domain C proteins from snakes (B) and other reptiles (C) to EBOV GP<sub>CL</sub> was determined by ELISA.